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(54) Title: STABILIZATION OF ENZYMES

(57) Abstract: Enzyme-containing formulations having improved stability and enzymatic activity in liquid medium, comprising one or more protease enzymes produced from any Bacillus bacteria, at least about 5 % alkali metal halide salt, and at least about 50 % polyol. Also disclosed are methods for making such formulations.



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STABILIZATION OF ENZYMES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to enzyme compositions and liquid formulations including enzymes. Particularly, the invention relates to methods for stabilizing enzymes and to liquid formulations with the stabilized enzymes.

2. Background

The stabilization of enzymatic activity is a standing problem in all areas of technology where enzymes are likely to be applied. Stability in this sense includes resistance to decrease in enzymatic activity prior to usage, e.g., under storage conditions. Stabilization of enzymes in liquid formulations is particularly a problem. For example, a pre-formulated liquid enzyme concentrate may sometimes be stored for weeks or months before eventually being blended into a final product (e.g., a personal care product, such as a hand cream; or a cleaning product, such as a liquid detergent). Similarly, formulated liquid products containing enzymes may sit in storage for lengthy periods of time before use, as well. For a variety of reasons, the activity of enzymes in liquid formulations typically decreases over time.

The prior art has attempted to deal with this problem. For example, organic compounds, such as sodium formate, and propylene glycol or glycerol are often added to liquid enzyme formulations. U.S. Pat. No. 4,318,818, Letton et al., issued March 9, 1982, discloses liquid detergents containing enzymes and an enzyme-stabilizing system comprising calcium ions and a low molecular weight carboxylic acid or salt, preferably a formate. The composition preferably contains an anionic surfactant and a saturated fatty acid. U.S. Pat. No. 4,404,115, Tai, issued September 13, 1983, discloses cleaning compositions containing enzymes, alkali metal pentaborate, alkali metal sulfite and a polyol. While somewhat effective, additives such as formate and other organic salts unfortunately are costly and, thus, significantly add to the expense of the liquid enzyme concentrate and final product. Further, there is in some instances a desire for formulations which are acceptable for food, pharmaceutical, or cosmetic use, and certain salts like sodium formate may not be acceptable or suitable for this purpose.

The use of inorganic salts as stabilization agents for enzymes is known in the art. For instance, U.S. Pat. No. 5,460,658, Nakagawa et al, issued October 24, 1995, discloses an enzyme based contact lens cleaning solution said to be stabilized with a polyhydric alcohol (5-30%) and alkali metal salt (1-5%) combination. The Nakagawa et al. patent teaches that polyhydric alcohol levels are less than 30% to maintain enzyme activity in the contact lens solution application. Xylanase was stabilized with a polyol (20-40%) and formate or potassium chloride (4-8%) as described in a study entitled "Development of A Method for the Stabilization and Formulation of Xylanase from *Trichoderma* Using Experimental Design", R. Spencer Fisk and Curran Simpson, as reported in Studies in Organic Chemistry, vol. 47, Stability and Stabilization of Enzymes, Elsevier, edited by W.J.J. Van Den Tweel, A. Harder, R.M. Buitelaar, 1992. This study discloses the use of no more than 40% polyol to maintain xylanase activity.

A cosmetic formulation having an enzyme stabilized using a 30-99% water-binding polyol that is partially or totally complexed with acrylic or methacrylic polymer is described in U.S. Pat. No. 5,830,449, Afriat et al., issued November 3, 1998, and U.S. Pat. No. 5,703,041, Afriat et al., issued December 30, 1997. The cosmetic formulation may also include an inorganic salt (2 to 12%) as a secondary stabilizing agent.

Clearly, there is a continuing need for liquid formulations that contain enzymes which are stabilized and exhibit a high activity over time. Particularly, there is a need for protease stabilized liquid formulations that are easy to process, highly effective, inexpensive to produce relative to the previously used stabilizing formulations, relatively inactive until ultimately included as an ingredient in a selected application, and also useful for formulations that are well tolerated physiologically.

SUMMARY OF THE INVENTION

It has been discovered that the combination of a high level of alkali metal halide salts, such as sodium chloride (at least 4% or 5% w/w, or higher), in combination with a polyol solvent, such as glycerol, propylene glycol, sucrose, etc (30% w/w, or higher), provides a high level of thermal stability for protease (produced from *Bacillus* species, e.g., subtilisin) formulations.

One aspect of the present invention provides a method for stabilizing one or more enzymes in a liquid medium. In one embodiment, the method of the invention

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includes the step of formulating in the liquid medium a high level of an alkali metal halide salt in combination with a polyol solvent.

The alkali metal halide salt can be, for example, sodium chloride, potassium chloride, and sodium or potassium fluoride or bromide. Lithium salts may also be
5 used. Additionally, other inorganic salts, such as salts of sulfates or sulfites, carbonates, phosphates, silicates or nitrates may be used. One preferred embodiment contemplates at least between 4-12% (e.g., at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, and/or at least 12%) sodium chloride. In one embodiment, the sodium chloride comprises between about 5-12%,
10 preferably about 8 - 12% w/w, of the liquid formulation.

The polyol can be present, for example, at a level of at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, and/or at least 95%. For example, one embodiment contemplates between 50 to 98% polyol, preferably between 55 and 95% polyol, preferably between 80 to 90% polyol, and most
15 preferably 50 to 80% polyol. Suitable polyols include, for example, glycerol, propylene glycol, sucrose, among others.

Among the various enzymes that can be stabilized by the teachings herein, one preferred enzyme is a protease, produced from *Bacillus* species, such as a subtilisin.

The liquid medium of the invention can be, for example, a liquid enzyme
20 concentrate or a formulated product including at least one enzyme, such as a protease. In one embodiment, the liquid medium is a formulated product selected from the group consisting of personal care products, health care products, and cleaning/detergency products. The liquid medium can be used, for example, in formulating hand creams, liquid detergents, and the like.

25 Another aspect of the present invention provides a liquid formulation providing an enzyme-stabilizing environment. In one embodiment, the formulation includes one or more enzymes, a high level of an alkali metal salt, and a polyol solvent.

The alkali metal halide salt can be, for example, sodium chloride, potassium chloride, and sodium or potassium fluoride or bromide. Lithium salts also may be
30 used. Additionally, other inorganic salts, such as salts of sulfates or sulfites, carbonates, phosphates, silicates or nitrates may be used. In one embodiment, the sodium chloride is present at a level of between 4-12% (e.g., at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, up to and

including 12%). In an exemplary formulation, the sodium chloride comprises about 8-12% w/w of the liquid formulation.

The polyol is preferably present, for example, at a level of at least 30%. Certain embodiments contemplate a polyol level of at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, and/or at least 95%. One embodiment contemplates between 50 to 98% polyol, preferably between 55 and 95% polyol, preferably between 80 to 90% polyol, and most preferably 50 to 80 polyol. Suitable polyols include, for example, glycerol, propylene glycol, sucrose, among others.

Among the one or more enzymes that are stabilized in the formulation, one embodiment contemplates inclusion of a protease produced from Bacillus species, preferably a subtilisin.

In one embodiment, the liquid formulation is a liquid enzyme concentrate or a formulated product including at least one enzyme, such as a protease produced from *Bacillus* species, such as a subtilisin. The liquid formulation can be, for example, a formulated product such as a personal care product, health care product, a cleaning product, a detergency product, among others.

Still a further aspect of the present invention provides a stabilized liquid enzyme formulation, including: (i) a protease, such as subtilisin; (ii) at least 5% w/w of an alkali metal halide salt, such as sodium chloride, potassium chloride, sodium or potassium fluoride or bromide, or lithium salts. Additionally, other inorganic salts, such as salts of sulfates or sulfites, carbonates, phosphates, silicates or nitrates may be used; and (iii) at least 30% w/w of a polyol solvent, such as glycol. Preferably, the formulation includes a subtilisin which is stabilized to exhibit at least about 98% activity remaining after 22 days at 37 degrees C. In one embodiment, the stabilized subtilisin includes an amino acid substitution at position 217 (e.g., Y217L).

These and other features, aspects and advantages of the present invention will become apparent from the following detailed description, in conjunction with the appended claims.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for stabilizing enzymes and liquid formulations including stabilized enzymes utilizing a polyol and an alkali metal halide salt.

5 Percentages herein are expressed as a percentage by weight of the liquid formulation.

The inventors hereof have determined that the combination of a high level of an alkali metal halide salt (e.g., sodium chloride) in combination with a polyol solvent (e.g., glycol) provides a high level of thermal stability for liquid enzyme (e.g., proteases
10 produced from *Bacillus* species) formulations.

Generally, formulations of the present invention include at least about 30% polyol or polyhydric alcohol; for example between from about 50 to about 98%, between from about 50 to about 95%, between from about 80 to about 90% polyol, and/or between from about 50-80% polyol. An exemplary liquid formulation suitable
15 for inclusion in personal care applications, as contemplated herein, includes between from about 50% to about 80% glycerol. Other suitable polyols include, and are not limited to, propylene glycol, ethylene glycol, sorbitol, mannitol, erythritol, dulcitol and inositol. Such a liquid formulation can be further formulated into, for example, a hand cream, or the like. A liquid formulation for liquid detergent applications, as
20 contemplated herein, includes between from about 33 to about 40% propylene glycol. Another exemplary liquid formulation for personal care application includes about 60% glycerol. The polyol level is approximately 50-80% to produce a compound having relatively inactive enzymes until such compound ultimately is included in a specific application.

25 Formulations of the present invention include at least about 5% of an alkali metal halide salt, such as sodium chloride, potassium chloride, and sodium or potassium fluoride or bromide, or lithium salts. Additionally, other inorganic salts, such as salts of sulfates or sulfites, carbonates, phosphates, silicates or nitrates may be used. In one preferred embodiment, the alkali halide metal salt is sodium chloride. The
30 sodium chloride can be present, for example, at a level of at least about 5%, at least about 6%, at least about 7%, at least about 8%, at least about 9%, at least about 10%, at least about 11%, and/or at least about 12%. In one embodiment, a liquid enzyme formulation includes between from about 5% to about 12% sodium chloride (e.g.,

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preferred at about 8% sodium chloride). In another preferred embodiment, the liquid enzyme formulation includes about 10% sodium chloride.

Preferred enzymes include those enzymes capable of hydrolyzing substrates. Such enzymes, which are known as hydrolases, include, but are not limited to, proteases (bacterial, fungal, acid, neutral or alkaline), amylases (alpha or beta), lipases, cellulases and mixtures thereof. Preferred proteases are also those described in US Re. 34,606 and EP 0 130 756, EP 251,446, EP 451,244, and EP 775,749, and incorporated herein by reference. Other preferred proteases are described in U.S. Pat. Nos. 6,218,165, 5,284,791, 5,700,676, 5,185,258, 5,155,033, 5,346,823, 5,763,257 and U.S. Pat. App. Ser. No. 09/768,080, filed February 8, 2000, titled Proteins Producing An Altered Immunogenic Response And Methods Of Making And Using The Same, describing protease mutants having an altered T-cell epitope. One preferred protease is available under the trade name Multifect, from Genencor International, Inc. Other preferred proteases include Purafect and Properase, from Genencor International, Inc. or Savinase, Esperase, Alcalase available from Novozymes A/S. Preferred proteases are those produced from any Bacillus species and include mutants which retain their bacillus protease-like structure and function. Other enzymes that can be used in the present invention include oxidases, peroxidases, transferases, dehydratases, reductases, hemicellulases and isomerases, among others. One or more enzymes may be included in the formulations of the present invention.

The stabilized liquid enzyme formulations of the present invention can be applied in a variety of fields, including the fields of personal care (e.g., protease for use in hand creams, and the like), and cleaning (e.g., protease for use in liquid detergents, and the like), among others. For example, the liquid enzyme formulations can be liquid enzyme concentrates which are useful for further formulation into final products, and/or they can be final formulated products, such as skin creams, lotions, liquid soaps, liquid detergents, etc.

Liquid enzyme formulations of the present invention are particularly effective at stabilizing enzymes during storage, within a temperature range of between from about 20 to about 40 degrees Celsius, e.g., at or around room temperature (22-25 degrees Celsius).

EXAMPLES

The following examples are illustrative and are not intended to limit the invention.

Example 1

5 Subtilisin BPN' Y217L was formulated at 5-7 gE/l in a base of 80-90% glycerol, 1 mM KH₂PO₄ at pH 5.0. The method of formulation generally involves the following: the 1mM CaCl₂ reagent was added to the glycerol reagent followed by the addition of the selected enzyme. Water was then added and the resulting mixture was
10 allowed to dissolve overnight at 4° C. The selected salt concentration was prepared by dissolving the salt in 1M NaOAC prior to addition to the dissolved enzyme. Next, 3% CP carbon was added to the enzyme salt combination and the resulting compound was mixed at 22° C for approximately 6 hours. Next, the compound was filtered and the resulting solution was stored for the stability studies set out below.

15 As shown in Table 1, the formulation was tested without further stabilization and with stabilization utilizing three stabilizers (sodium acetate, sodium chloride, and sodium formate) .

TABLE 1: Stability Study 1**Basic Conditions for all samples*** 50 ppm Ca²⁺

10 g enzyme solution

* Temperature 25C/37C

Experiments				Product Activity
B0	KH ₂ PO ₄ 10 mM @ pH 5.0 before additions; 10%	Sodium Acetate; 8%	Glycerol; 82%	6.75 gE/l
B1	KH ₂ PO ₄ 10 mM @ pH 5.0 before additions; 10%	Sodium Chloride; 8%	Glycerol; 82%	6.75 gE/l
B2	KH ₂ PO ₄ 10 mM @ pH 5.0 before additions; 10%	Sodium Formate; 8%	Glycerol; 82%	6.75 gE/l
B3	KH ₂ PO ₄ 10 mM @ pH 5.0 before additions; 10%	None	Glycerol; 90%	6.75 gE/l
B4	KH ₂ PO ₄ 10 mM @ pH 5.0 before additions; 10%	None	Glycerol; 90%	3.37 gE/l
B5	KH ₂ PO ₄ 10 mM @ pH 5.0 before additions; 10%	None	Glycerol; 45% / Propylene Glycol; 45%	6.75 gE/l
B6	KH ₂ PO ₄ 10 mM @ pH 5.0 before additions; 10%	None	Propylene Glycol; 90%	6.75 gE/l
B7	KH ₂ PO ₄ 10 mM @ pH 5.0 after additions; 10%	None	Glycerol; 90%	6.75 gE/l
B8	KH ₂ PO ₄ 10 mM @ pH 5.5 before additions; 10 %	None	Glycerol; 90%	6.75 gE/l

Activity levels for all of the above formulations were monitored as exhibited by the data of Table 2, which demonstrates that sodium chloride is a very effective agent for stabilization of subtilisin (note, in particular, B1). Remaining enzyme activity at 22 days utilizing the three stabilizers as shown was between 96-98% as compared to a value of only 81% in the remaining formulations that did not include sodium acetate, sodium chloride or sodium formate.

Table 2: 37 °CSubtilisin Stability StudyActivities by AAPF-pNA Assay in $\Delta\text{AU min}^{-1}\text{ml}^{-1}$

Day	Buffer								
	0	1	2	3	4	5	6	7	8
0	206	211	233	229	112	234	204	200	204
1	212	216	239	238	113	238	214	171	214
6	205	210	228	214	98	217	177	161	198
22	196	206	224	185	49	179	109	142	160
41	186	208	212	150	11	163	62	116	138
66	186	205	202	129	7	132	32	33	113
94	183	192	183						88
Day	Activity left % Buffer								
	B0	B1	B2	B3	B4	B5	B6	B7	B8
0	100	100	100	100	100	100	100	100	100
1	103	102	102	104	101	102	105	85	105
6	100	100	98	94	88	93	87	80	97
22	96	98	96	81	43	77	54	71	78
41	91	99	91	66	10	70	30	58	67
66	91	97	87	57	6	57	16	16	55
94	89	91	79						43

The 8% NaCl sample was found to have high stability (over 98 % activity remaining after 22 days at 37 degrees C and 100% after 23 days at 37 degrees C.). The data further demonstrates that a high activity level (at least 90%) remained in the presence of sodium chloride out to 94 days.

Table 3 below represents the same example as above conducted at 25° C.

Table 3: 25 °CSubtilisin Stability StudyActivities by AAPF-pNA Assay in $\Delta\text{AU min}^{-1}\text{ml}^{-1}$

Day	Buffer								
	0	1	2	3	4	5	6	7	8
0	206	211	233	229	112	234	204	200	204
2	205	216	223	227	108	227	200	184	197
23	204	212	242	214	94	223	188	166	203
30	200	213	220	214	85	215	182	153	197
65	201	206	227	192	40	187	158	135	174
94	189	197	222						158
Day	Activity left % Buffer								
	B0	B1	B2	B3	B4	B5	B6	B7	B8
0	100	100	100	100	100	100	100	100	100
2	100	102	96	99	97	97	98	92	96
23	99	101	104	93	84	95	93	83	99
30	97	101	94	93	76	92	90	77	97
65	98	98	97	84	35	80	78	68	77
94	92	93	95						77

Example 2 illustrates the results of a study designed to increase the commercial desirability of liquid enzyme formulations and to facilitate manufacturing of such formulations. Accordingly, Example 2 illustrates the surprising results achieved by reducing the concentration of glycerol and increasing the concentration of salt in the formulation.

Subtilisin BPN' Y217L was formulated as set out in Table 4 below in a base of 70-82% glycerol, 1 mM KH₂PO₄.

TABLE 4: Stability Study 2

Basic Conditions for all samples

* 50 ppm Ca²⁺

10 g enzyme solution

* Temperature 25C/37C

Form #	Enzyme Buffer	Salt** (g)	Salt Type	Glycerol** (g)	Target Enzyme Activity (mg/mL)	Enzyme (wt/wt %)	Final Form Density
B1	10 mM K-Phos, ph 5.0	8	NaOAC	82	6.75	0.54	1.25
B2	10 mM K-Phos ph 5.0	8	NaCl	82	6.75	0.54	1.25
B3	10 mM K-Phos ph 5.0	8	NaOAC.3H ₂ O	82	1.3	0.104	1.25
B4	10 mM K-Phos ph 5.5	12	NaOAC.3H ₂ O	70	1.3	0.104	1.25
B5	10 mM K-Phos ph 5.5	8	NaCl	82	1.3	0.104	1.25
B6	10 mM K-Phos ph 5.5	6	NaCl	82	1.3	0.104	1.25
B7	10 mM K-Phos ph 5.5	8	NaCl	70	1.3	0.104	1.25
B8	10 mM K-Phos ph 5.5	10	NaCl	60	1.3	0.104	1.25
B9	100 mM K-Phos ph 5.5	6	NaCl	82	1.3	0.104	1.25
B10	100 mM K-Phos ph 5.5	8	NaCl	82	1.3	0.104	1.25
B11	100 mM K-Phos ph 5.5	8	NaCl	70	1.3	0.104	1.25

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B12	100 mM K-Phos ph 5.5	10	NaCl	60	1.3	0.104	1.25
B13	100 mM K-Phos ph 5.5	8	NaOAC.3H ₂ O	82	1.3	0.104	1.25
B14	100 mM K-Phos ph 5.5	12	NaOAC.3H ₂ O	70	1.3	0.104	1.25

As shown by the surprising data of Table 5 below, a stable glycerol/salt formulation that is easily manufactured may be achieved by increasing the salt concentration thereby enabling a reduction in the amount of glycerol in the formulation.

Table 5 illustrates that a 60% glycerol and 10% salt formulation is exceptionally stable.

TABLE 5

Subtilisin Stability Study: 25°C

Activities by AAPF-pNA Assay in $\Delta\text{AU min}^{-1}\text{ml}^{-1}$

Buffer														
Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	323	327	63.3	63.7	63.8	66.4	63.4	66.5	65.3	66.9	65.4	63.5	64.9	63.2
118	310	327	63.6	61.4	66.9	60.9	61.7	66.9	60.9	63.2	66.4	64.4	63.8	62.6
174	320	336	65.9	63.7	62.6	59.1	63.6	68.6	61.7	64.6	64.1	66.8	65.6	63.7
279	301	301	57.7	59.9	51.8	50.8	62.6	62.1	53.8	54.8	59.9	59.4	57.4	59.0

10

% Original Activity														
	Buffer													
Day	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
118	96.0	100.0	100.5	96.4	104.8	91.6	97.3	100.7	93.3	94.5	101.6	101.4	98.2	99.0
174	99.2	102.6	104.2	100.1	98.1	88.9	100.2	103.1	94.6	96.5	98.0	105.1	101.1	100.9
279	93.2	91.8	91.2	94.0	81.2	76.5	98.6	93.3	82.5	81.9	91.6	93.5	88.4	93.4

15

TABLE 6

37 C

Activities by AAPF-pNA Assay in $\Delta\text{AU min}^{-1}\text{ml}^{-1}$

	Buffer													
Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	323	327	63.3	63.7	63.8	66.4	63.4	66.5	65.3	66.9	65.4	63.5	64.9	63.2
9	313	316	63.1	64.0	64.3	64.2	62.7	63.0	61.6	64.1	60.7	61.4	62.4	61.2
29	311	320	63.3	63.2	64.1	63.4	62.3	64.4	60.4	64.3	61.1	61.7	62.7	61.4
112	269	298	57.9	55.3	62.4	48.4	64.7	63.4	48.9	62.2	60.9	58.9	57.4	56.5

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173	281	233	54.7	50.3	57.3	12.4	61.7	64.6	27.7	60.7	60.7	61.7	55.9	52.6
280	242	43.9	48.3	44.6	6.79	4.42	56.9	58.3	4.76	32.3	52.5	55.5	47.1	42.4
% Original Activity														
	Buffer													
Day	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
9	97.0	96.6	99.8	100.5	100.7	96.6	98.9	94.7	94.4	95.8	92.8	96.7	96.2	96.8
29	96.4	97.7	100.0	99.2	100.4	95.4	98.1	96.9	92.5	96.0	93.4	97.2	96.7	97.1
112	83.4	90.9	91.4	86.9	97.8	72.9	102.0	95.4	75.0	93.0	93.2	92.7	88.4	89.4
173	87.0	71.2	86.4	79.0	89.8	18.7	97.3	97.1	42.5	90.7	92.9	97.2	86.1	83.3
280	75.1	13.4	76.3	70.0	10.6	6.6	89.6	87.7	7.3	48.3	80.3	87.4	72.6	67.1

Protease Assay

Examples 1 and 2 measured activity utilizing a protease assay with N-succinyl-L-Ala-L-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (suc-AAPF-pNA). The assay is based upon the cleavage by proteases of the amide bond between phenylalanine and *p*-nitroaniline of the N-succinyl reagent. *P*-nitroaniline is monitored spectrophotometrically at 410 nm and the rate of the appearance of *p*-nitroaniline is a measure of proteolytic activity. A protease unit is defined as the amount of protease enzyme that increases absorbance at 410 nm by 1 absorbance unit (AU)/min of a standard solution of 1.6 mM suc-AAPF-pNA in 0.1 M Tris Buffer at 25°C in a cuvet with a 1cm path length. The glycerol was obtained from JT Baker.

Various other examples and modifications of the foregoing description and examples will be apparent to a person skilled in the art after reading the disclosure without departing from the spirit and scope of the invention, and it is intended that all such examples or modifications be included within the scope of the appended claims. All publications and patents referenced herein are hereby incorporated by reference in their entirety.

CLAIMS

1. A method for stabilizing one or more protease enzymes produced from *Bacillus* species in a liquid medium, comprising:

formulating in said liquid medium a high level of an alkali metal halide salt in combination with a polyol solvent.

2. The method of claim 1, wherein said alkali metal halide salt comprises sodium chloride, said sodium chloride being present at a level of at least about 4%, preferably at least about 5%, and most preferably between from about 5% to about 12%.

3. The method of claim 1, wherein said polyol is present at a level of at least about 50%, preferably between from about 50 to about 80%.

4. The method of claim 1, wherein said polyol solvent is selected from the group consisting of glycerol, propylene glycol, sucrose, and any combination thereof.

5. The method of claim 1, wherein said one or more protease enzymes is a subtilisin.

6. The method of claim 1, wherein said liquid medium is a liquid enzyme concentrate or a formulated product.

7. The method of claim 6, wherein said liquid medium is a formulated product selected from the group consisting of personal care products, health care products, cleaning products, and detergency products.

8. A liquid formulation providing an enzyme-stabilizing environment, said formulation including one or more proteases produced from a *Bacillus* species, a high level of an alkali metal halide salt, and a polyol solvent.

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9. The formulation of claim 8, wherein said alkali metal salt comprises sodium chloride, said sodium chloride being present at a level of at least about 4%, preferably at least about 5%, and most preferably between from about 5% to about 12%.

10. The method of claim 8, wherein said polyol is present at a level of at least about 50%, preferably between from about 50 to about 80%.

11. The formulation of claim 10, wherein said polyol solvent is selected from the group consisting of glycerol, propylene glycol, sucrose, and any combination thereof.

12. The formulation of claim 8, wherein said one or more protease enzymes includes a subtilisin.

13. The formulation of claim 8, wherein said liquid formulation is a liquid enzyme concentrate or a formulated product.

14. The formulation of claim 13, wherein said liquid formulation is a formulated product selected from the group consisting of personal care products, health care products, cleaning products, and detergency products.

15. A stabilized liquid enzyme formulation, including:
- (i) a protease produced from any *Bacillus* species,
 - (ii) at least about 5% of an alkali metal halide salt, and
 - (iii) at least about 50% of a polyol solvent.

16. The formulation of claim 15, wherein said alkali metal salt comprises sodium chloride.

17. The formulation of claim 15, wherein said polyol solvent comprises propylene glycol.

18. The formulation of claim 15, wherein said protease has at least about 95% activity remaining, preferably at least about 98% activity remaining, after 22 days at 37 degrees C.

19. The formulation of claim 15, wherein said protease is a subtilisin.

20. The formulation of claim 19, wherein said subtilisin includes an aminoacid substitution at position 217.

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(54) Title: STABILIZATION OF ENZYMES

(57) Abstract: Enzyme-containing formulations having improved stability and enzymatic activity in liquid medium, comprising one or more protease enzymes produced from any Bacillus bacteria, at least about 5 % alkali metal halide salt, and at least about 50 % polyol. Also disclosed are methods for making such formulations.

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INTERNATIONAL SEARCH REPORT

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 3 296 094 A (THEODORE CAYLE) 3 January 1967 (1967-01-03)	1-4, 6, 8-11, 13
Y	column 2, paragraph 3; claims 1, 3 ---	1-20
Y	WO 00 12663 A (ALCON LAB INC) 9 March 2000 (2000-03-09) the whole document ---	1-20
X	US 5 759 984 A (SHETTY JAYARAMA K ET AL) 2 June 1998 (1998-06-02)	1, 2, 4-9, 12-14
Y	the whole document ---	3, 10, 11, 15-20
Y	GB 1 232 627 A (PACIFIC BIOCHEMICAL) 19 May 1971 (1971-05-19) the whole document ---	1-20
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 169 817 A (WEBER MEYER M) 2 October 1979 (1979-10-02)	1, 2, 4-9, 12-14
Y	the whole document	1-20
Y	US 5 269 960 A (GRAY ROBERT L ET AL) 14 December 1993 (1993-12-14) column 7, paragraph 4 column 9, line 2	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/23076

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 3296094	A	03-01-1967	NONE	
WO 0012663	A	09-03-2000	US 6184189 B1 US 6214596 B1 AU 4955199 A WO 0012663 A1	06-02-2001 10-04-2001 21-03-2000 09-03-2000
US 5759984	A	02-06-1998	AU 714742 B2 AU 3934395 A CA 2213122 A1 EP 0795009 A1 FI 972792 A WO 9620274 A1 JP 10511549 T NO 973008 A NZ 295735 A	13-01-2000 19-07-1996 04-07-1996 17-09-1997 27-08-1997 04-07-1996 10-11-1998 27-08-1997 29-06-1999
GB 1232627	A	19-05-1971	NONE	
US 4169817	A	02-10-1979	US B458819 I5	13-04-1976
US 5269960	A	14-12-1993	NONE	